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Stearidonic acid, a plant-based dietary fatty acid, enhances the chemosensitivity of canine lymphoid tumor cells

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ABSTRACT

Lymphoma is the most common hematopoietic tumor in dogs and humans, with similar pathogenesis and therapeutic responses. Anticancer drugs like vincristine (VCR) and doxorubicin (DOX) are often used in treating lymphoma. However, the cure rate is generally poor due to chemoresistance. Here, we sought to determine whether stearidonic acid (SDA), a plant-based dietary fatty acid, sensitizes chemoresistant canine lymphoid-tumor cells. GL-1 B-cell lymphoid-tumor cells were found to be highly sensitive to the antitumor-activity of VCR and DOX, while OSW T-cell and 17-71 B-cell lymphoid-tumor cells were moderately and fully resistant, respectively. SDA, at its non-toxic concentrations, significantly promoted the antitumor action of VCR and DOX in both OSW and 17-71 cells. SDA-mediated chemosensitization was associated with SDA inhibition of P-glycoprotein (P-gp) function. This was confirmed in HEK293 cells stably expressing P-gp as well as by increased binding-affinity of SDA to P-gp in P-gp docking analysis. SDA at its chemosensitizing concentrations did not affect the viability of healthy dog peripheral blood mononuclear cells, suggesting that SDA is non-toxic to normal dog peripheral blood leucocytes at its chemosensitizing concentrations. Our study identifies a novel dietary fatty acid that may be used as a dietary supplement in combination with chemotherapy to promote the antitumor efficacy of the chemotherapy drugs in dogs and possibly in humans with chemoresistant lymphoma.

1. Introduction

Both human and canine lymphomas have similar clinical presentation, biological behavior, and response to chemotherapy [1–4]. In dogs, lymphoma is one of the most common cancers accounting for up to 25% of all canine cancers [5]. Both B-cell and T-cell lymphomas can affect any dog of any breed at any age [6]. Most untreated dogs diagnosed with malignant lymphoma generally survive less than 6 weeks [7]. Lymphoma is usually treated with aggressive chemotherapy protocols involving a combination of chemotherapeutics, including vincristine (VCR) and doxorubicin (DOX) [8–10]. While these multi-agent chemotherapy regimens improve the survival time, relapses are frequently seen [7]. Most importantly, relapsed lymphoma often displays chemoresistance [11,12], resulting in a poor prognosis.

Chemoresistance in a variety of human cancers is associated with upregulation of expression/function of multidrug transporters, particularly drug efflux pumps such as P-glycoprotein (P-gp) [13]. Likewise, chemoresistance in canine lymphoma was shown to be associated with P-glycoprotein (P-gp) [14]. Hence, there is a need to identify novel therapeutic approaches to combat chemoresistance by inhibiting the expression/activity of P-gp.

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Dietary fatty acids could significantly improve the activity of chemotherapy drugs in cancer cells by downregulating multidrug transporters. For instance, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) greatly enhance the antitumor activity of DOX or paclitaxel in chemoresistant human colon cancer cells by reducing the expression and activity of P-gp and other drug efflux pumps [15,16]. Similarly, DHA improves the antitumor activity of DOX in DOX-resistant human breast cancer cells by suppressing the expression of P-gp [17].

Recently, a plant-based stearidonic acid (SDA), which serves as an alternative source to fish-based fatty acids, has been shown to exert antitumorigenic effects in human cancers [18,19]. However, the chemosensitizing nature of SDA is unknown. In the current study, we investigated whether SDA can potentiate the antitumor activity of VCR and/or DOX in the chemoresistant canine lymphoid tumor cells, and whether SDA can inhibit P-gp activity at its chemosensitizing concentrations.

2. Materials and methods

2.1. Cell culture

Canine OSW T-cell and GL-1 and 17-71 B-cell lymphoid tumor cell lines were grown in RPMI-1640 medium (Lonza) supplemented with 10% FBS (HyClone). HEK293/pcDNA3 and HEK293/ABCB1 (HEK293 cells stably expressing P-gp) [20] were grown in DMEM (Lonza). The cells were cultured in an incubator with a humidified atmosphere maintained at 5% CO_2 and 95% air at 37°C. The lymphoma cell lines are well characterized to reflect *in vivo* properties and are well-established models for lymphoma studies [2,21,22].

2.2. Isolation of dog peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from mixed breed dogs, maintained in breeding colonies at Auburn University. All procedures performed with the dogs were approved by the Auburn University IACUC. Briefly, up to 50 ml of blood was collected by venipuncture, typically of the jugular vein, and was placed into tubes containing EDTA to prevent clotting. The blood was layered onto an equal volume of Ficoll (1.077) and centrifuged at 400 × G for 30 min. The opaque interface was collected and washed with isotonic phosphate buffered saline and then centrifuged at 250 × G for 10 min. The supernatant was carefully aspirated and the pellet was washed a second time using the same method. The pellet was then resuspended in RPMI media and cultured as described above.

2.3. Materials

Stearidonic acid (SDA) (Cayman Chemical) was reconstituted in ethanol (ETOH). Puromycin (PUR) (Cellgro) was reconstituted in PBS. Doxorubicin (DOX), Vincristine (VCR), Valspodar (PSC-833), and Rhodamine 123 (R123) were purchased from Sigma and reconstituted in dimethyl sulfoxide (DMSO).

2.4. Cell viability assays

The lymphoid tumor cells or healthy dog peripheral blood mononuclear cells were plated into 96-well culture plates (PerkinElmer) at a density of 10,000 cells per well. The cells were then either untreated or treated with PBS, DMSO, ETOH, VCR, DOXO, SDA ± VCR, or SDA ± DOX for 24 h. Next, the CellTiter-Glo luminescent cell viability assays (Promega) were performed to determine the number of viable cells by quantifying the ATP present, which indicates the presence of metabolically active cells [23,24]. Luminescence was measured with a FLUOstar Optima microplate reader (BMG Labtech).

2.5. Intracellular rhodamine 123 accumulation assays

The efflux activity of P-glycoprotein (P-gp) was determined by measuring the intracellular accumulation of the fluorescent P-gp probe rhodamine 123 (R123) [23]. Briefly, the cells were washed with HBBS (without Ca^{2+}, Mg^{2+} and phenol red) and incubated at 37 °C for 15 min with or without DMSO, ETOH, SDA or PSC-833 (P-gp specific inhibitor) in HBBS. R123 (5 μM) was added after 15 min to the cells in the presence or absence of DMSO, ETOH, SDA or PSC-833 and incubated for another 45 min. The cells were washed with ice-cold-HBBS and solubilized in Triton-HBBS. To determine the intracellular concentration of R123, the fluorescence was measured using Infinite microplate reader (TECAN) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.6. Molecular modeling studies

The structure of SDA was built using the builder module of Maestro v 9.3.5 and subsequently energy minimized by Macromodel program v9.9 (Schrödinger, Inc., New York, NY, 2012) using the OPLSAA force field with the steepest descent followed by truncated Newton conjugate gradient protocol. The X-ray crystal structure of P-gp in apoprotein state (PDB ID: 3G5U) and in complex with inhibitors QZ59-RRR (PDB ID: 3G60) or QZ59-SSS (PDB ID: 3G61; obtained from the RCSB Protein Data Bank) were used to build the homology model of human P-gp as previously described [20,25,26]. The diverse conformational library of SDA was docked at each of the generated grids (sites-1–4 of P-gp) using the "Extra Precision" (XP) mode of Glide program v5.8 (Schrödinger, Inc., New York, NY, 2012) with the default functions. The top scoring conformations of SDA with P-gp were used for graphical analysis.

2.7. Data presentation and statistical analysis

Cell viability data are expressed as percentage of untreated cells (control), where control was set as 100% viability. The fluorescence intensity of the samples without R123 was considered as the background. Fluorescence intensity of all the samples with R123 was subtracted with the background fluorescence before data normalization. The efflux activity of P-gp is presented as relative R123 accumulation by normalizing the fluorescence intensity of the samples with ETOH, SDA or PSC-833 to the samples without ETOH, SDA or PSC-833. The unpaired Student's t-test was used to determine statistical significance ($^*p < 0.05$).

3. Results and discussion

3.1. Canine lymphoid tumor cells are resistant to chemotherapy drugs

Several multi-agent chemotherapy protocols, consisting of VCR and DOX, are clinically employed to treat dogs with lymphoma [8–10]. However, relapses associated with chemoresistance are often seen in the lymphoma patients [14]. We examined whether canine lymphoid tumor cells are resistant to VCR and/or DOX using cell viability assays. VCR and DOX significantly decreased the viability of GL-1 B-cell lymphoid tumor cells in a concentration-dependent manner (Fig. 1A and B). On the other hand, both VCR and DOX showed little and moderate effect on the viability of and 17-71 B-cell and OSW T-cell lymphoid tumor cells, respectively (Fig. 1A and B). Together, these results suggest that GL-1 cells are

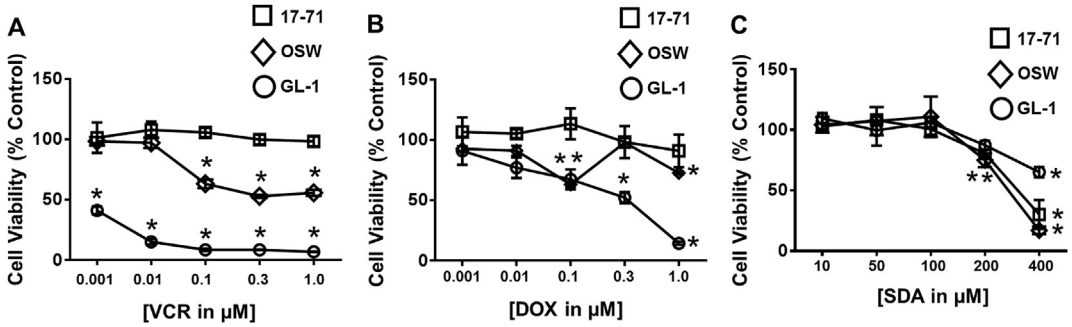

Fig. 1. Effect of VCR, DOX, and SDA on viability of canine lymphoid tumor cells: Cell viability was measured after treating 17-71, GL-1, and OSW cells with vehicles DMSO, ETOH, or with increasing concentrations of VCR (A), DOX (B), and SDA (C) as indicated for 24 h. Data are shown as mean ± SD after normalizing with untreated control. *, $p < 0.05$; compared with the untreated control.

sensitive to VCR or DOX treatment, while OSW and 17-71 cells, respectively, are moderately and fully resistant to VCR or DOX.

3.2. SDA chemosensitizes canine lymphoid tumor cells

Some dietary fatty acids, including SDA have been shown to exert antitumor actions in human cancers [18,19]. We investigated whether SDA affects the viability of the lymphoid tumor cells. Although SDA induced cytotoxic effect at higher concentrations (200 and 400 μM), it did not significantly affect the viability of the lymphoid tumor cells up to 100 μM (Fig. 1C).

It is evident from combination chemotherapeutic approaches that some dietary fatty acids, promote therapeutic efficacy of chemotherapy drugs [15–17]. We tested whether SDA at its non-cytotoxic concentrations can enhance the antitumor activity of VCR/DOX in the chemoresistant lymphoid tumor cells. Indeed, SDA at 50 and 100 μM significantly promoted the antitumor action of VCR (Fig. 2A) and DOX (Fig. 2B) in 17-71 and OSW cells. These results suggest that SDA enhances the sensitivity of the chemoresistant lymphoid tumor cells towards VCR and DOX.

Notably, SDA at 100 μM did not affect the viability of healthy dog peripheral blood mononuclear cells (Fig. 2C). Although VCR and DOX displayed marginal cytotoxicity in peripheral blood mononuclear cells, SDA at 100 μM did not potentiate the cytotoxic effect of VCR or DOX (Fig. 2C). These observations suggest that SDA could be non-cytotoxic to normal peripheral blood leucocytes at its chemosensitizing concentrations.

3.3. SDA increases intracellular R123 accumulation in canine lymphoid tumor cells

Very recently, it was shown in DOX-resistant canine lymphoid tumor cells that Masitinib, a tyrosine kinase inhibitor, inhibits the function of P-gp, suggesting that chemoresistance can be reversed in canine lymphoid tumor cells by inhibiting the function of P-gp [27]. SDA potentiation of the antitumor activity of VCR and DOX (Fig. 2A and B) could be a consequence of suppression of chemoresistance. Since VCR and DOX are known substrates of P-gp, we logically speculated that SDA may chemosensitize the lymphoid tumor cells by inhibiting the efflux activity of P-gp [28,29]. We therefore determined whether SDA alters the efflux activity of P-gp in the lymphoid tumor cells.

PSC-833, a prototypical inhibitor of P-gp, significantly increased the intracellular accumulation of P-gp substrate R123 in the chemoresistant 17-71 and OSW cells but not in the chemosensitive GL-1 cells (Fig. 3). These data suggest that only the chemoresistant 17-71 and OSW cells but not chemosensitive GL-1 cells express functional P-gp. Absence of functional P-gp system in GL-1 cells is in

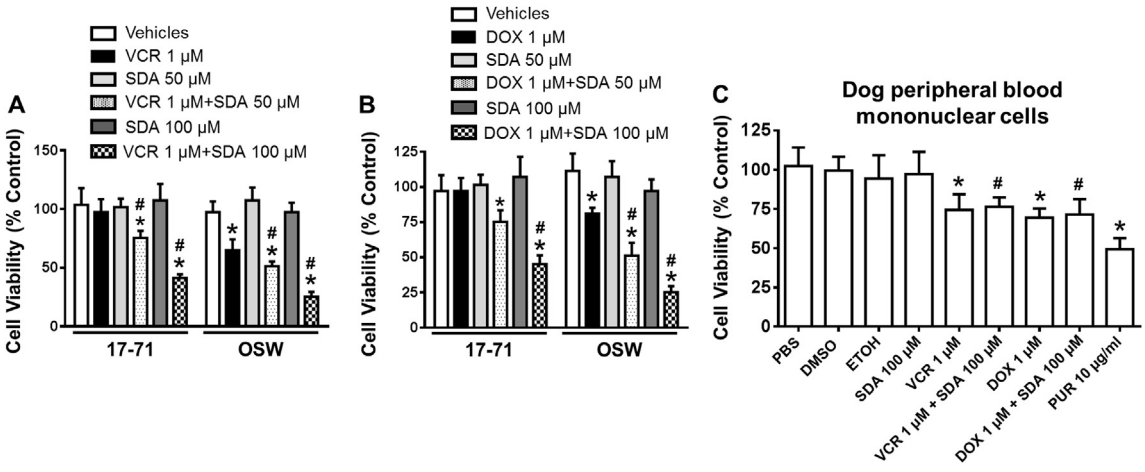

Fig. 2. Chemosensitizing effect of SDA in chemoresistant canine lymphoid tumor cells, and effect SDA on viability of healthy dog peripheral blood mononuclear cells: Cell viability was measured after treating 17-71 and OSW cells with the vehicles (DMSO + ETOH), VCR ± SDA (A) or DOX ± SDA (B) as indicated for 24 h. Data represent mean ± SD from three independent experiments. *, $p < 0.05$, by comparing VCR, DOX, SDA, VCR + SDA, or DOX + SDA in each cell line with the untreated control. #, $p < 0.05$, by comparing VCR + SDA or DOX + SDA with VCR or DOX alone in each cell line. (C) Healthy dog peripheral blood mononuclear cells were treated with PBS, DMSO, ETOH, SDA, or PUR (positive control cytotoxic agent) as indicated for 24 h. Cell viability was measured after the treatments and the data were normalized to the untreated controls. The data represent mean ± SD of six observations. *, $p < 0.05$; compared with the untreated control.

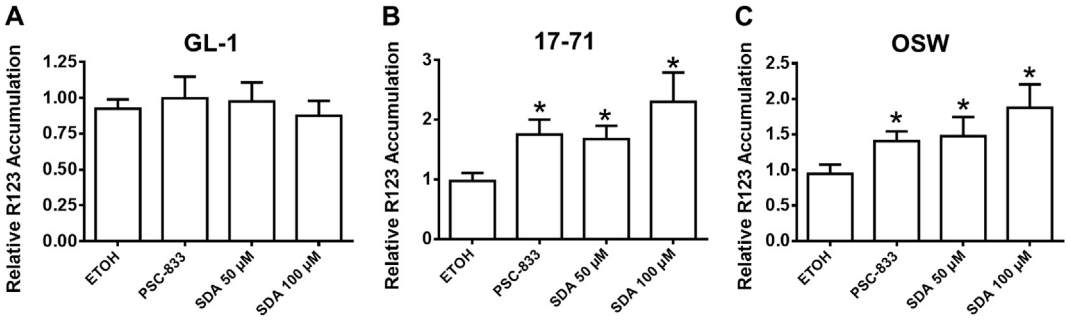

Fig. 3. Effect of SDA on intracellular R123 accumulation in canine lymphoid tumor cells: R123 fluorescence was measured in GL-1 (A), 17-71 (B) and OSW (C) cells in the absence or presence of DMSO, ETOH, SDA, or P-gp specific inhibitor PSC-833. Relative R123 accumulation was determined by normalizing the fluorescence in the absence or presence of DMSO, ETOH, SDA, or PSC-833, as described in the methods. The data are presented as mean ± SD of four independent experiments ($^*p < 0.05$).

agreement with a previous report [27] and is also consistent with lack of resistance towards VCR and DOX induced cytotoxicity (Fig. 1). Similar to PSC-833, SDA, at its non-toxic chemosensitizing concentrations (50 and 100 μM), considerably increased the intracellular accumulation of R123 in the chemoresistant OSW and 17-71 but not in the chemosensitive GL-1 cells (Fig. 3), suggesting that SDA inhibits the efflux activity of P-gp.

To confirm whether SDA inhibits the efflux activity of P-gp, we measured the intracellular R123 accumulation in HEK293/pcDNA3 and HEK293/ABCB1 cells. Similar to PSC-833, SDA, at its non-toxic chemosensitizing concentrations, increased the intracellular R123 accumulation in HEK293/ABCB1 cells that stably express P-gp (Fig. 4B) but not in HEK293/pcDNA3 cells (Fig. 4A). The fact that SDA, similar to PSC-833, increased the intracellular rhodamine 123 in the chemoresistant 17-71 and OSW cells, but not in the chemosensitive GL-1 cells (Fig. 3), suggests that the effect of SDA is specific in the chemoresistant cells and points to an underlying mechanism of SDA-mediated chemosensitization by inhibiting P-gp efflux activity.

3.4. Binding model of SDA to P-gp

Molecular modeling studies were performed to understand the binding mechanism of SDA to P-gp protein. The XP-Glide predicted binding mode of SDA at site 1 is shown in Fig. 4C. As illustrated in the schematic diagram (Fig. 4D), SDA binds to active site 1 of P-gp through extensive van der Waal's interactions and one-hydrogen bonding interaction. A substructural component (C5 to C11) of SDA which includes two alkenyl groups (C6 and C9) and three methylene spacers (C5, C7 and C11) adopts a 'W' like configuration

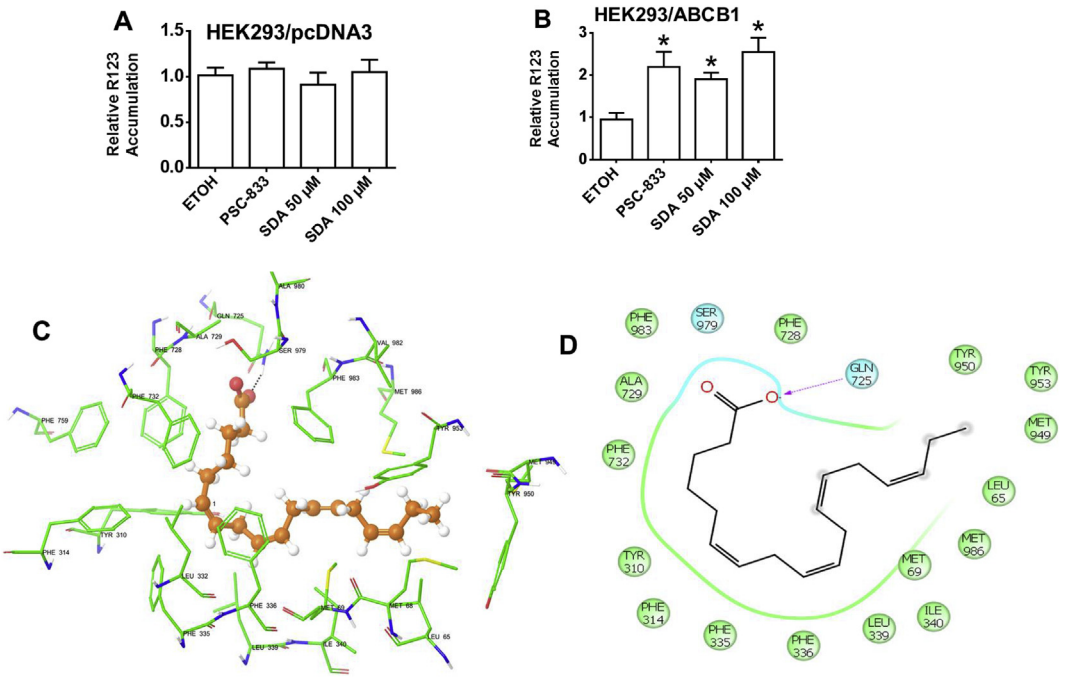

Fig. 4. Effect of SDA on intracellular R123 accumulation in HEK293/pcDNA3 and HEK293/ABCB1 cells, and binding model of SDA to P-gp: R123 fluorescence was measured in HEK293/pcDNA3 (A) and HEK293/ABCB1 (B) cells in the absence or presence of DMSO, ETOH, SDA, or P-gp specific inhibitor PSC-833. Relative R123 accumulation was determined by normalizing the fluorescence in the absence or presence of DMSO, ETOH, SDA, or PSC-833. The data are presented as mean ± SD of four independent experiments ($^*p < 0.05$). Binding model of SDA to P-gp: (C) XP-Glide predicted binding mode of SDA with homology modeled ABCB1. The docked conformation of SDA as ball and stick model is shown within the large hydrophobic cavity of ABCB1. Important amino acids are depicted as sticks with the atoms colored as carbon – green, hydrogen – white, nitrogen – blue, oxygen – red, sulfur – yellow, whereas SDA is shown with the same color scheme as above except carbon atoms are represented in orange. Dotted black line indicates hydrogen-bonding interactions. (D) Schematic diagram of the protein–ligand interaction is shown for the SDA. Blue circles code polar amino acids; hydrophobic amino acids by green circles and purple arrows depict side chains' donor/acceptor interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inside the hydrophobic pocket I at site-1 establishing a series of hydrophobic interactions with amino acid residues Phe314, Phe315, Phe336, Leu339 and Ileu340 surrounding the hydrophobic pocket. This 'W' like core also appears to anchor the flexible side chain containing the carboxyl moiety and the semi rigid side chain with a terminal ethyl group in the binding site-1. The semi rigid side chain consisting of two alkenyl groups and a terminal ethyl group is positioned in the second hydrophobic pocket flanked by Leu65, Met949, Tyr953, Tyr950, Met986, Phe732 whereas the flexible side chain with a carboxyl moiety is oriented towards a polar sub pocket made of three amino acid residues (Gln725, Ala729 and Ser979) favoring a hydrogen bonding interaction between oxygen of carboxylate group and NH of carboxamide side chain Gln725. Furthermore, the carbonyl oxygen of the carboxyl moiety is also favorably oriented towards the OH of Ser979 indicating the possibility of electrostatic interactions between them. This specific spatial orientation of carboxyl moiety is reinforced by favorable hydrophobic interactions between the methylene groups in the flexible side chain and aromatic amino acids Phe728, Phe732, Phe983 bordering the active site-1. Together, increased binding affinity of SDA at P-gp binding site explains as how SDA could block the functions of P-gp in effluxing its substrate anticancer agents such as VCR and DOX.

An ideal plant-based dietary supplement would be one that promotes the antitumor efficacy of chemotherapy drugs in the chemoresistant canine lymphoid tumor cells, with limited cytotoxicity. The fact that SDA, at its chemosensitizing concentrations, was non-cytotoxic to healthy dog peripheral blood mononuclear cells (Fig. 2C) indicates that SDA could be a safe *in vivo* dietary supplement. Whether SDA will have similar chemoadjuvant properties *in vivo* needs to be determined. Similar dietary fatty acids such as EPA and DHA in humans could even attain a concentration of 500 μM in plasma after oral intake in diet [30,31]. Additionally, these fatty acids exhibited no noticeable adverse effects at these plasma concentrations. Conceivably, the chemosensitizing concentration of SDA in dogs can be accomplished and this scenario is even more plausible with the availability of genetically modified SDA-rich soybeans [32–34]. This, however, needs to be tested.

Canine cancer models are emerging as a powerful tool for advancing human oncology studies [35,36]. This is primarily because dogs share similar environment with humans and harbor naturally-occurring cancers with characteristics in many ways identical or similar to human cancers [35,36]. It is important to note that canine lymphoma has been reported as a good model for studying both pathogenesis and treatment of human lymphoma since both canine and human lymphoma share similar characteristics in disease development and response to chemotherapy [1–4]. Therefore, our results from canine lymphoma cells are highly relevant to human lymphoma.

To our knowledge, this is the first report that SDA, at its non-cytotoxic concentrations, could sensitize the chemoresistant canine lymphoid tumor cells to the chemotherapy drugs. Additionally, we showed for the first time that SDA could inhibit P-gp function at clinically achievable concentrations. Nevertheless, other possible mechanisms of chemosensitization should not be overlooked and evaluated in future studies. For example, SDA may interact with other transporters such as MRPs or BCRP. Also, SDA may inhibit activity of NF-kB signaling as chemoresistance in canine lymphoma is also associated with the presence of constitutive canonical NF-kB activity [1]. The beneficial chemosensitizing effects of SDA may allow it to be used as a chemoadjuvant to help prevent relapses of lymphoma in dogs and improve clinical outcome. In conclusion, our results suggest that SDA could be used as a dietary supplement in combination chemotherapy with VCR and DOX, and possibly with other chemotherapeutic drugs.

Conflict of interest

None.

Acknowledgments

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